ORIGINAL ARTICLE

Title: Effects of tea extracts on the colonization behaviour of *Candida* species: attachment inhibition and biofilm enhancement

Running title: Tea modulates *Candida* colonization

Yi Wang[1], H.M.H.N. Bandara[1], Deirdre Mikkelsen[2], Lakshman P. Samaranayake[1]*

[1] School of Dentistry, University of Queensland, Brisbane, Australia.

[2] The University of Queensland, ARC Centre of Excellence in Plant Cell Walls, Centre for Nutrition and Food Sciences, Queensland Alliance for Agriculture and Food Innovation, Brisbane, Queensland 4072, Australia.

Correspondence:

Lakshman P. Samaranayake, School of Dentistry, University of Queensland, 288 Heston Road, Herston, Queensland, Australia.

Email: l.samaranayake@uq.edu.au

Tel: +601 7 3365 8062

Word Count: 3848

Abstract

Purpose. We assessed the effects of four different types of tea extracts (green, oolong, black and pu-erh tea) on cellular surface properties (hydrophobicity and auto-aggregation) and the colonization attributes (attachment and biofilm formation) of four strains of *Candida albicans* and three strains of *Candida krusei*.

Methodology. The cellular surface properties were determined using spectrophotometry. The colonization activities were quantified using colorimetric viability assays and visualized using scanning electron microscopy and confocal laser scanning microscopy.

Results. The tea extracts, in general, reduced the hydrophobicity (by 8-66%) and auto-aggregation (by 20-65%), and inhibited the attachment of two *C. krusei* strains (by 41-88%). Tea extracts enhanced the biofilm formation of one *C. albicans* and two *C. krusei* strains (by 1.4-7.5 folds). The observed reduction in hydrophobicity strongly correlated with the reduction in attachment of the two *C. krusei* strains ($p<0.05$). The ultrastructural images of the tea-treated *C. krusei* biofilm cells demonstrated central indentations, though remained viable.

Conclusion. The tea extracts have the ability to retard *C. krusei* adhesion to glass surfaces possibly by reducing fungal cellular hydrophobicity, whilst paradoxically promoting biofilm formation. In practical terms, therefore, consumption of tea beverages appears to have a complex effect on oral candidal colonisation.

Keywords: *Candida*; tea; cell surface hydrophobicity; microbial attachment; biofilm.

Abbreviations: HIV, human immunodeficiency virus; SDA, sabouraud dextrose agar; SDB, sabouraud dextrose broth; BATH, bacterial attachment to hydrocarbon; OD, optical density; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide; SEM, scanning electron microscopy; CLSM, confocal laser scanning microscopy.

Introduction

Candida spp. are oral inhabitants of approximately 50% of the human population [1]. These microbes are considered important opportunistic pathogens as they frequently cause infections in compromised individuals, such as those on chemotherapy and HIV-infected individuals [2], and organ transplant recipients on immunosuppressives [3]. In general, *Candida albicans* is the most common oral species, whilst others such as *Candida glabrata, Candida tropicalis, Candida krusei* and *Candida guilliermondii* are less abundant, though consistently isolated [1]. Indeed, in some communities, *C. krusei* is the most prevalent *Candida* species isolated from the oral cavity [4].

Upon gaining access to the oral milieu, *Candida* spp. colonize the mucosal surfaces and abiotic surfaces such as prosthesis, including dental implants, and survive essentially as biofilms, which in essence exhibit greater resistance to host defences and antifungal agents than their planktonic counterparts [2, 5, 6]. Auto-aggregation of *Candida* cells (blastospores), their co-aggregation with other oral microorganisms, as well as their attachment to dental hard and soft tissues, are the prerequisites for successful colonization and biofilm formation [7-9]. It is well known that the attachment of microorganisms to different surfaces (or to each other) involves surface physico-chemical interactions such as hydrophobic, electrostatic, and steric [10-13]. For example, attachment of oral streptococci to abiotic surfaces correlates well with their cell surface hydrophobicity [14], as well as their cell surface charge [15]. In the cases of fungi, especially yeasts which do not possess cell surface appendages and are therefore more similar to colloidal particles, physico-chemical interactions are likely to play an important role in the attachment and biofilm formation [16].

Traditional therapies for microbial infection are currently challenged due to their potential undesirable side effects, as well as emergence of antimicrobial resistance,

particularly in biofilm-related diseases [17]. Natural chemicals have therefore been of great interest, and extensively studied as novel agents to prevent *Candida* infections. Tea (*Camellia sinensis*), after water, is the second most popular drink worldwide [18] and its impact on oral candidal colonisation, including biofilm formation, has been little studied. To date, the majority of research on tea and its by-products has focused on its antimicrobial, including anti-candidal activities [19-23]. Yet, the impact of tea on candidal attachment and biofilm formation has not been extensively studied. We previously reported that tea extracts exhibited the ability to prevent *Streptococcus mutans* from attaching and forming biofilms on different abiotic surfaces, due to a superficial coat of tea components on the bacterial surfaces [13]. Similar investigations, to our knowledge, have yet to be performed with *Candida* spp. despite the fact that yeasts are key constituents of the oral microbiome and are often found to influence the colonization of other oral bacteria [8, 24].

Therefore, in this study, we hypothesized that tea extracts affected the colonization behaviour of *Candida* spp. in a physico-chemical manner, akin to that of *Streptococcus mutans*, as demonstrated previously [13]. The aims of the current study, therefore, were to determine *in vitro* the impact of the extracts from four commercial tea products (with increasing degree of fermentation) on: i) the attachment and biofilm formation; and ii) the physico-chemical properties of seven different *Candida* strains belonging to *C. albicans* (four strains) and *C. krusei* (three strains). Furthermore, we aimed to correlate the physico-chemical properties with the colonization potential of tea-treated *Candida* species.

Materials and methods

Microbial cultures

Four strains of C. *albicans* (strain 1, SF1, E1 200/5/92 and ATCC 90028), along with three strains of *C. krusei* (strain CamL 27B, CamL 37B and ATCC 6258) were used in this study. All *Candida* strains, except the ATCC strains, are clinical isolates, and were obtained from the *Candida* collection at the Oral Bisocience Laboratories, at the Faculty of Dentistry, University of Hong Kong. All strains were maintained on sabouraud dextrose agar (SDA; Sigma-Aldrich, USA) at 4°C, and grown in sabouraud dextrose broth (SDB; Sigma-Aldrich, USA) at 37°C under agitation (150 r.p.m.) for 24 h. Microbial cell suspensions were prepared by centrifuging 20 ml of SDB cultures at 3000 g for 5 min. Thereafter, the pellets were washed with 150 mM PBS (2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, and 137 mM NaCl, pH 7.4; Sigma-Aldrich, USA), and resuspended in 20 ml PBS for all experiments, unless otherwise stated.

Preparation of tea extracts

Extracts of four commercial tea products, namely green tea, oolong tea, black tea and pu-erh tea (T2 Co. Ltd, Australia; country of origin of the tea leaves: China), were prepared by mixing each tea with distilled water, at a 1/20 (w/v) ratio, for 24 h [25] at 37°C. Thereafter, the mixture was filtered, and the filtrate was evaporated under vacuum at 40°C, freeze dried and stored at -20°C for further use. The temperatures used in all steps were kept at or below 40°C, as a higher temperature would destroy tea polyphenols.

Stock solutions of the tea extracts were prepared by dissolving 100 mg of the extract in 10 ml of PBS and filter sterilized through a 0.2 μm filter.

Antimicrobial susceptibility tests

The minimal inhibitory concentrations (MICs) of the tea extracts against the *Candida* strains were determined using the micro-broth dilution method as previously described by James [26]. Briefly, 100 µl of each filter sterilized tea extract solution (at a final concentration of 20 mg ml^{-1}) was subject to double dilution in a microtitre plate, mixed with 100 µl of SDB containing suspended *Candida* cells (approximately 10^4 c.f.u. ml^{-1}), and incubated at 37°C for 24 h. Growth was determined by visually assessing the turbidity in the wells. In subsequent experiments, each strain was treated with tea extracts at the concentration below the lowest MIC value among all tea extracts, to make the studies comparable. According to the results of the antimicrobial susceptibility tests, a concentration at 10 mg ml^{-1} was chosen for all tea extracts, for use in all subsequent assays.

Determination of cell surface hydrophobicity

The cell surface hydrophobicity was determined using the Bacterial Attachment to Hydrocarbon (BATH) method as previously described by Wang *et al.* [27], with the following modifications. Briefly, cell suspensions containing dissolved tea extracts were adjusted to OD_{595} = 1.0±0.2. Controls were prepared by using PBS without tea extracts, and using tea extracts-PBS solution without *Candida* cells. A 3 ml aliquot of each sample was mixed with 1 ml of hexane and vortexed for 2 min. The mixture was allowed to phase separate for 1 h. The OD_{595} of the aqueous layer was measured before (A_0) and after (A) the addition of hexane. The cell surface hydrophobicity was expressed as % of binding to hexane = $(1–A/A_0) \times 100$ %.

Auto-aggregation assays

Auto-aggregation measurements were performed as described by Wang *et al.* [27]. A volume of 1 ml of cell suspension (with or without tea extracts) was adjusted to OD_{595} = 0.25±0.05 prior to incubation at 37°C for 6 h. The OD_{595} was measured before (A_i) and after (A_f) the incubation. Aggregation percentage was expressed as % of auto-aggregation = (1–A_f/A_i) × 100 % [28, 29].

Preparation of glass beads

Glass beads (4 mm; Eureka Beads, Australia) were degreased by soaking in acetone for 1h, washed with 0.1 M HCl for 30 min, followed by 0.1 M NaOH for 30 min, and rinsed in distilled water for 30 min, prior to autoclaving. Sterilized beads were oven dried overnight and thereafter appropriately stored for use in attachment experiments (and biofilm formation assays).

Microbial attachment assays

Microbial attachment assays were performed on prepared glass beads (as described above), using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reduction assay [30]. Briefly, a glass bead with 100 µl of cell suspension (at 10^7 c.f.u. ml^{-1}; with or without tea extracts), was incubated in a single well of a microtitre plate at 37°C for 1 h with shaking at 80 r.p.m.. After incubation, the bead was removed from the well, gently washed three times with PBS to remove loosely attached cells, placed in a well of another microtitre plate containing 79 µl PBS, 20 µl XTT solution (1 mg ml^{-1}; Sigma-Aldrich, USA) and 1 µl of fresh prepared menadione solution (0.4 mM; Sigma-Aldrich, USA), and further incubated in the dark for 3h at 37°C. Thereafter, the bead was removed from the well and colour changes of the solution in the well were measured using a microtitre

plate reader (Spectra Max 340 tunable microplate reader; Molecular Devices Ltd, Sunnyvale, CA) at 492 nm.

Biofilm assays

Biofilm formation assays were performed on prepared glass beads using the XTT reduction assay [30]. Briefly, a glass bead with 50 µl of tea extract (at a final concentration of the sub-MIC) and 50 µl double concentrated SDB culture (10^5 c.f.u. ml^{-1}) was incubated in a well of a microtitre plate at 37°C for 48h with shaking at 80 r.p.m.. Controls were prepared by using distilled water instead of tea extract solutions. After incubation, the beads were treated as described in the microbial attachment assay protocol above.

Scanning electron microscopy (SEM) study

The biofilm structures, cell distributions and the surface topography of tea treated and untreated samples were visualized using SEM. The untreated and tea treated *Candida* biofilms were grown on glass slides (1cm × 1cm) by immersing a slide in 10 ml SDB culture and incubating in a falcon tube at 37°C for 48 h. After incubation, the slides were washed in PBS, air dried and fixed with 4% (v/v) glutaraldehyde (Sigma-Aldrich, USA) in PBS. The fixed slides were washed again in PBS, frozen in a -80°C freezer and freeze dried [13]. The slides were then gold-sputtered and examined using a field emission scanning electron microscope (Carl Zeiss Inc., Oberkocken, Germany) at 15 kV and an 8.4 mm working distance.

Confocal laser scanning microscopy (CLSM) study

The viability of tea treated and untreated biofilm cells was assessed using CLSM. The untreated and tea treated *Candida* biofilms were grown on glass cover slips (1cm × 2cm) as described in the SEM study protocol above. After incubation, the slides were stained with SYTO® 9 dye and Propidium iodide (Live/Dead BacLight Bacterial Viability kit; Invitrogen, Eugene, OR, USA) [31] prior to visualization using a Nikon C2 confocal laser scanning microscope (Nikon Corp., Tokyo, Japan). The CLSM study was undertaken only for the non-fermented green tea extract and post-fermented pu-erh tea, in order to compare the effects of monomeric and polymeric tea polyphenols.

Statistical analysis

All assays were carried out in triplicate with independently grown cultures, and all values were expressed as mean ± standard deviation. A one way ANOVA (Tukey's comparison) was performed to compare the control and treatments in each assay. The relationships between cell surface properties and their colonization abilities were determined using regression plots. All data expressed as percentage values were normalized by arcsine-transformation. All analysis were conducted using the SPSS software (PASW Statistics 18; SPSS Inc.) at a 95% confidence level.

Results

Antimicrobial susceptibility tests

The results of antimicrobial susceptibility tests indicated that the tea extracts at 20 mg ml^{-1} could not kill or inhibit any of the *Candida* spp. (data not shown). Therefore, a concentration of 10 mg ml^{-1} (a non-lethal dose) was chosen and used for all tea extracts in all

assays in order to ensure that the effects of the tea extracts on the properties and colonization behaviour of *Candida* spp. could be tested without either killing or inhibiting the cells.

Determination of cell surface properties and colonization behaviour

The results of the cell surface hydrophobicity assays are shown in Fig. 1. It was found that the tea extracts significantly reduced the cell surface hydrophobicity of most of the tested *Candida* strains ($p<0.05$) by 8-66%, except for *C. krusei* ATCC 6258 ($p>0.05$), whose hydrophobicity was not reduced by any of the tea extracts. Of all the tea extracts tested, the pu-erh tea extract was the most effective in reducing the hydrophobicity ($p<0.05$), by 27-66% reduction in hydrophobicity.

Similarly to the results obtained for the hydrophobicity assays, the tea extracts significantly reduced the auto-aggregation of all *Candida* strains, in most of the cases ($p<0.05$) by 20-65%, except *for C. krusei* ATCC 6258 ($p>0.05$) (Fig 2).

The results from the microbial attachment assays indicated that the tea extracts could only inhibit the attachment to glass beads of the *C. krusei* strains 27B and 37B ($p<0.05$), by 41-88% (Fig. 3). However, the biofilm assays indicated that the tea extracts did not inhibit the biofilm formation of most of the *Candida* spp. ($p>0.05$), yet enhanced the biofilm production by 1.4-7.5 folds for *C. albicans* strain ATCC 90028 and for *C. krusei* strains ATCC 6258 and 37B ($p<0.05$) (Fig. 4). *C. krusei* strain 37B was therefore selected for subsequent microscopy studies.

Correlations between cell surface properties and colonization behaviours

The correlation between the changes in cell surface properties (hydrophobicity and auto-aggregation) and the changes in colonization behaviours (attachment and biofilm formation), due to the tea extract treatments, were determined using a regression plot (Fig. 5). A significant positive correlation was observed between the changes in cell surface hydrophobicity and the changes in attachment of *C. krusei* strains 27B (Fig. 5A) and 37B (Fig. 5B). Regression values (R^2) of 0.724 and 0.799 were observed respectively ($p<0.05$ for both strains), suggesting that the reduction in hydrophobicity could be a mechanism underlying the attachment inhibitory effect of the tea extracts on *C. krusei* (strains 27B and 37B). A similar correlation was not observed for other strains ($p>0.05$). No correlation was observed between auto-aggregation and attachment/biofilm formation for all strains ($p>0.05$).

Microscopy

The SEM and CLSM images of the untreated and tea-treated *C. krusei* 37B cells are shown in Fig. 6. Although multiple SEM micrographs and CLSM images were taken, only one representative micrograph or image is presented here per treatment (Fig. 6, A-G). While there were no significant morphologic differences observed between the tea extract-treated and untreated cells in the attachment assays (images not shown), a marked difference was observed for the tea-treated and untreated cells in the biofilms. Despite all treated and untreated cells were processed in the same manner to avoid sample preparation biases, the tea extract-treated biofilm cells appeared to form denser clusters, and presented with a dent or pock mark in the middle of each cell (Fig. 6 C and D). However, the untreated cells appeared to be intact and formed relatively smaller clusters (Fig. 6 A and B). As this phenomenon was observed with *Candida* treated with all the tested tea extracts, only the images of green tea treated cells are shown (Fig. 6). Furthermore, the live/dead CLSM images indicated that the

pock-marked, indented cells treated with green tea were all viable (Fig. 6F) unlike those treated with the pu-erh tea extract, where a scant distribution of non-viable cells was observed within the biofilm matrix (Fig. 6G).

Discussion

The results from the antimicrobial susceptibility tests indicated that none of the crude tea extracts evaluated exerted any antifungal effect on the tested *Candida* spp., as they appeared not to kill or inhibit the yeasts at a relatively high tea concentration (20 mg ml^{-1}). The choice of whole crude tea extracts instead of isolated tea compounds could be a possible reason for our findings, which contradict those of recent research, where a strong fungicidal effect has been reported [16, 20, 21]. However, our data imply that the crude polyphenols or their derivatives in tea are unlikely to inhibit or kill oral *Candida*, due to their relatively low concentrations, as well as the high temperature that would also destroy the tea compounds [25]. Nevertheless, we demonstrate here that ordinary drinking tea is likely to alter *Candida* colonisation of the oral cavity in a species and strain dependent manner.

The results obtained from the surface property assays showed that the four tea extracts reduced cell surface hydrophobicity and their ability to auto-aggregate. One could speculate that the tea components present in these extracts may have affected the cell surfaces by possibly binding physically or chemically to the yeast blastospore surface, thereby altering their cell surface properties. We have previously observed this phenomenon for *Streptococcus mutans*, a major cariogenic organism commonly found in the oral cavity [13]. In the latter study, we observed that tea polyphenols (especially tannins) coating the cells of *Streptococcus mutans* suppressed their adhesion, as well as biofilm formation on three different abiotic surfaces: glass, stainless steel, and hydroxyapatite - the major constituent of

tooth enamel. Furthermore, this veneer of `tea coating` was also visible through electron microscopy. However, in the present study, no such visible alterations of the cell surfaces of *C. krusei* were observed after tea-extract treatment (Fig. 6).

As the tea components were extracted using water, it could be surmised that most of the extracts were highly polar and were able to interfere with the hydrophobicity of the cell surfaces. According to the interfacial thermodynamic theory [32], the reduced hydrophobicity may result in weaker hydrophobic interactions between the cells and the substratum surface, as well as between different cells, which in turn would reduce the auto-aggregation, and thus, in theory, the attachment of the cells to the contact surface. The findings of our study fit the foregoing model very well as we observed a very high correlation between reduction in hydrophobicity and attachment due to tea extract exposure (R^2 at 0.724 and 0.799).

However, this was not the case for the attachment of all *C. albicans* strains tested, and for some strains of *C. krusei*. Clearly, hydrophobic interactions alone may not entirely explain the key mechanisms that mediate the attachment of these strains. Microbial attachment is often deemed as a two-step process. Physico-chemical interactions usually dominate the initial step, helping the cells to approach the contact surface and loosely attach to it. In the second step, cell surface proteins/adhesins play their role in helping the cells to firmly stick to the surface [33]. Hence, for those strains whose attachment could not be affected by hydrophobic interactions, other physico-chemical factors, such as electrostatic interactions or non-physico-chemical factors, such as cell surface adhesins, might play the major role in their attachment behaviour [34].

As for *C. krusei* strains 27B and 37B, the reduction in hydrophobicity eventuated by the tea extract treatment strongly correlated with the reduction in attachment, indicating that hydrophobic interactions may be the key factor affecting their attachment. In other words,

306 reducing the hydrophobic interactions in the system could possibly control the initial
307 colonization of these *C. krusei* strains. However, the attachment of the type-culture *C. krusei*
308 strain (ATCC 6258) was not affected by the tea extracts, suggesting that the yeast may have
309 altered cell surface components. It is known that type culture strains, such as *C. krusei*
310 ATCC 6258, which are repeatedly sub-cultured in the laboratory over a prolonged period,
311 loose their cell surface attributes in comparison to their wild-type counterparts [1]. The latter
312 phenomenon may account for the disparate behaviour of *C. krusei* ATCC 6258 we observed.

313 As attachment is the first stage of biofilm formation, it would be reasonable to assume
314 that inhibiting attachment would reduce biofilms. Yet, the results obtained from the biofilm
315 assays in this study indicated otherwise. The tea extracts did not inhibit biofilm formation by
316 the tested *Candida* strains, but rather enhanced biofilm formation for some strains (*C.*
317 *albicans* strain ATCC 90028 and *C. krusei* strains ATCC 6258 and 37B), suggesting there
318 might be variables other than physico-chemical interactions affecting their biofilm formation,
319 such as chemical and biological factors. It could be speculated that the tea extracts might
320 have induced a chemical stress on the cells, thus impelling them to aggregate and form
321 thicker biofilms as a protective mechanism against this stress [35], which is evident in the
322 microscopic images where the tea extract treated cells formed denser biofilms. However, this
323 was not supported by the auto-aggregation assays conducted in this study probably due to the
324 different experimental conditions used in the auto-aggregation and biofilm assays such as
325 incubation duration (6h and 48h, respectively) and growth media (PBS and SDB,
326 respectively). Furthermore, it has been reported that tea polyphenols inactivate proteasomal
327 enzymes in *Candida* cells, and these enzymes are used by the cells to regulate metabolism
328 and respond to environmental signals [36]. Interfering with the functions of these enzymes
329 could affect cell behaviour, in terms of proliferation and forming biofilms. Evensen and
330 Braun [36] also reported that inactivating these enzymes inhibited biofilm formation by

Candida spp., but this was not the case here. A possible reason could be that the tea polyphenols used by Evensen and Braun were pure compounds at relatively high concentrations, while the present study used crude tea leaf extracts.

Interestingly, the microscopic images showed tea extract treated *C. krusei* blastospores with central indentations or pock marks, in spite of which the cells were viable, as observed by live/dead stain microscopy. One exception was the pu-erh tea treated samples, where a few non-viable cells were observed in the biofilm. Such findings could be explained in terms of the undissociated protons from the polyphenol molecules within the tea extract, causing cellular energy depletion with an increased ratio of ADP/ATP. This, in turn, may have inhibited DNA synthesis or arrested the process of cellular proliferation at the anaphase, a phenomenon previously reported by Tan *et al.* [37]. Thus, it is plausible that the cells with indentations could not yet complete the division due to this arrested development. This phenomenon has been observed and reported by other researchers in different microorganisms, such as *Salmonella* [37] and *Escherichia coli* [38].

In conclusion, the four extracts from green, oolong, black and pu-erh teas used in this study did not kill or inhibit the growth of the tested *Candida* strains, but inhibited the attachment of two strains of *C. krusei* to glass surfaces, possibly due to a reduction in cell surface hydrophobicity. However, the biofilm development of three of the *Candida* strains tested was enhanced by the tea extracts. There was also a simultaneous morphological change in the biofilm cells of *C. krusei,* leading to the formation of a central indentation in the cell walls of each blastospore. Therefore, in practical terms, while tea consumption may not necessarily kill oral *Candida* spp., it is likely to affect the initial colonization of oral surfaces by species such as *C. krusei,* while simultaneously enhancing their biofilm development.

Future studies need to focus on purifying specific compounds from tea extracts and evaluating the effect of the isolated compounds on *Candida* biofilm formation by a larger variety of *Candida* species, to determine the effectiveness as well as to expand the current knowledge on the effect of specific tea components on oral candidal colonization. Also, the removal effects of tea extracts/components on pre-formed oral biofilms should be investigated in addition to studying the biofilm prevention effects. Furthermore, the ADP/ATP ratio could be measured by bioluminescent assays in order to confirm the assumption of cellular energy depletion induced by the tea extracts.

Funding information

This work received no specific grant from any funding agency.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to acknowledge the Faculty of Dentistry, the University of Hong Kong, for providing the *Candida* strains.

References

1. **Samaranayake LP.** Host factors and oral candidosis. In: Samaranayake LP, MacFarlane TW (editors). *Oral candidosis*. London: Butterworth; 1990. pp. 66-103.
2. **Calderone RA**. *Candida and candidiasis*. Washington D. C.: ASM Press; 2002.

3. **Denning DW, Donnelly JP, Hellreigel KP, Ito J, Martino P *et al.*** Antifungal prophylaxis during neutropenia or allogeneic bone marrow transplantation: what is the state of the art? Ad HOC Working Group. *Chemotherapy* 1992;38 Suppl 1:43-49.
4. **Reichart PA, Samaranayake LP, Samaranayake YH, Grote M, Pow E *et al.*** High oral prevalence of *Candida krusei* in leprosy patients in northern Thailand. *J Clin Microbiol* 2002;40(12):4479-4485.
5. **Perlroth J, Choi B, Spellberg B.** Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med Mycol* 2007;45(4):321-346.
6. **Thein ZM, Seneviratne CJ, Samaranayake YH, Samaranayake LP.** Community lifestyle of *Candida* in mixed biofilms: a mini review. *Mycoses* 2009;52(6):467-475.
7. **Cannon RD, Chaffin WL.** Colonization is a crucial factor in oral candidiasis. *J Dent Educ* 2001;65(8):785-787.
8. **Grimaudo NJ, Nesbitt WE.** Coaggregation of *Candida albicans* with oral *Fusobacterium* species. *Oral Microbiol Immunol* 1997;12(3):168-173.
9. **Lamont R, Jenkinson H.** Adhesion as an ecological determinant in the oral cavity. In: Kuramitsu HK, Ellen RP (editors). *Oral bacterial ecology: the molecular basis*. Wymondham: Horizon Scientific Press; 2000. pp. 131-168.
10. **Busscher HJ, Weerkamp AH, van der Mei HC, van Pelt AW, de Jong HP *et al.*** Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. *Appl Environ Microbiol* 1984;48(5):980-983.
11. **Chen Y, Busscher HJ, van der Mei HC, Norde W.** Statistical analysis of long- and short-range forces involved in bacterial adhesion to substratum surfaces as measured using atomic force microscopy. *Appl Environ Microbiol* 2011;77(15):5065-5070.
12. **van der Mei HC, Roseberg M, Busscher HJ.** Assessment of microbial cell surface hydrophobicity. In: Mozes N, Handley PS, Busscher HJ, Rouxhet PG (editors). *Microbial cell surface analysis: structural and physicochemical methods*. New York: VCH Publishers; 1991. pp. 265-289.
13. **Wang Y, Lee SM, Dykes GA.** Potential mechanisms for the effects of tea extracts on the attachment, biofilm formation and cell size of *Streptococcus mutans*. *Biofouling* 2013;29(3):307-318.
14. **Nostro A, Cannatelli MA, Crisafi G, Musolino AD, Procopio F *et al.*** Modifications of hydrophobicity, in vitro adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysum italicum* extract. *Lett Appl Microbiol* 2004;38(5):423-427.
15. **Abbott A, Rutter PR, Berkeley RC.** The influence of ionic strength, pH and a protein layer on the interaction between *Streptococcus mutans* and glass surfaces. *J Gen Microbiol* 1983;129(2):439-445.
16. **Panagoda GJ, Ellepola AN, Samaranayake LP.** Adhesion of *Candida parapsilosis* to epithelial and acrylic surfaces correlates with cell surface hydrophobicity. *Mycoses* 2001;44(1-2):29-35.
17. **Sanguinetti M, Posteraro B, Lass-Florl C.** Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses* 2015;58 Suppl 2:2-13.
18. **Graham HN.** Green tea composition, consumption, and polyphenol chemistry. *Preventive medicine* 1992;21(3):334-350.
19. **Antunes DP, Salvia AC, de Araujo RM, Di Nicolo R, Koga Ito CY *et al.*** Effect of green tea extract and mouthwash without alcohol on *Candida albicans* biofilm on acrylic resin. *Gerodontology* 2015;32(4):291-295.
20. **Betts JW, Wareham DW, Haswell SJ, Kelly SM.** Antifungal synergy of theaflavin and epicatechin combinations against *Candida albicans*. *J Microbiol Biotechnol* 2013;23(9):1322-1326.
21. **Chen M, Zhai L, Arendrup MC.** *In vitro* activity of 23 tea extractions and epigallocatechin gallate against *Candida* species. *Med Mycol* 2015;53(2):194-198.
22. **Doddanna SJ, Patel S, Sundarrao MA, Veerabhadrappa RS.** Antimicrobial activity of plant extracts on *Candida albicans*: an *in vitro* study. *Indian J Dent Res* 2013;24(4):401-405.
23. **Sitheeque MA, Panagoda GJ, Yau J, Amarakoon AM, Udagama UR *et al.*** Antifungal activity of black tea polyphenols (catechins and theaflavins) against *Candida* species. *Chemotherapy* 2009;55(3):189-196.

24. **Jenkinson HF, Douglas LJ.** Interactions between *Candida* species and bacteria in mixed infections. In: Brogden KA, Guthmiller JM (editors). *Polymicrobial Diseases*. Washington D. C.: ASM Press; 2002. pp. 357-376.
25. **Perva-Uzunalić A, Škerget M, Knez Ž, Weinreich B, Otto F *et al.*** Extraction of active ingredients from green tea (*Camellia sinensis*): Extraction efficiency of major catechins and caffeine. *Food Chemistry* 2006;96(4):597-605.
26. **James PA.** Comparison of four methods for the determination of MIC and MBC of penicillin for viridans *streptococci* and the implications for penicillin tolerance. *J Antimicrob Chemother* 1990;25(2):209-216.
27. **Wang Y, Lee SM, Dykes GA.** Growth in the presence of sucrose may decrease attachment of some oral bacteria to abiotic surfaces. *Annals of Microbiology* 2015;65(2):1159-1163.
28. **Barki M, Koltin Y, Yanko M, Tamarkin A, Rosenberg M.** Isolation of a *Candida albicans* DNA sequence conferring adhesion and aggregation on Saccharomyces cerevisiae. *J Bacteriol* 1993;175(17):5683-5689.
29. **Shen S, Samaranayake LP, Yip HK.** Coaggregation profiles of the microflora from root surface caries lesions. *Arch Oral Biol* 2005;50(1):23-32.
30. **Bandara HM, BP KC, Watt RM, Jin LJ, Samaranayake LP.** *Pseudomonas aeruginosa* lipopolysaccharide inhibits *Candida albicans* hyphae formation and alters gene expression during biofilm development. *Mol Oral Microbiol* 2013;28(1):54-69.
31. **Jin Y, Zhang T, Samaranayake YH, Fang HH, Yip HK *et al.*** The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in *Candida albicans* biofilms. *Mycopathologia* 2005;159(3):353-360.
32. **Van Oss C, Good R, Chaudhury M.** The role of van der Waals forces and hydrogen bonds in "hydrophobic interactions" between biopolymers and low energy surfaces. *Journal of colloid and Interface Science* 1986;111(2):378-390.
33. **Alberti-Segui C, Morales AJ, Xing H, Kessler MM, Willins DA *et al.*** Identification of potential cell-surface proteins in *Candida albicans* and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. *Yeast* 2004;21(4):285-302.
34. **Wang Y, Lee SM, Dykes G.** The physicochemical process of bacterial attachment to abiotic surfaces: Challenges for mechanistic studies, predictability and the development of control strategies. *Crit Rev Microbiol* 2015;41(4):452-464.
35. **Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S *et al.*** Inhibitory effects of oolong tea extract on caries-inducing properties of *mutans streptococci*. *Caries Res* 1999;33(6):441-445.
36. **Evensen NA, Braun PC.** The effects of tea polyphenols on Candida albicans: inhibition of biofilm formation and proteasome inactivation. *Can J Microbiol* 2009;55(9):1033-1039.
37. **Tan SM, Lee SM, Dykes GA.** Acetic acid induces pH-independent cellular energy depletion in *Salmonella enterica*. *Foodborne Pathog Dis* 2015;12(3):183-189.
38. **Teh AH, Wang Y, Dykes GA.** The influence of antibiotic resistance gene carriage on biofilm formation by two *Escherichia coli* strains associated with urinary tract infections. *Can J Microbiol* 2014;60(2):105-111.

Figure Captions:

Fig. 1 The effect of the tea extracts on cell surface hydrophobicity of *C. albicans* and *C. krusei*. For each strain, the values labelled with dissimilar letters indicate significant differences in hydrophobicity due to different types of tea treatment ($p<0.05$). The statistical comparisons were based on arcsine-transformed data (n=3).

Fig. 2 The effects of the tea extracts on auto-aggregation of *C. albicans* and *C. krusei.* For each strain, the values labelled with dissimilar letters indicate significant differences in auto aggregation due to different types of tea treatment ($p<0.05$). The statistical comparisons were based on arcsine-transformed data (n=3).

Fig. 3 The effects of the tea extracts on attachment of *C. albicans* and *C. krusei* to glass surfaces (OD reading; n=3). For *C. krusei* 27B and 37B, the values labelled with dissimilar letters indicate significant differences in attachment due to different types of tea treatment ($p<0.05$). Significant differences in attachment to glass surfaces were not observed after tea treatment for the remaining strains.

Fig. 4 The effects of the tea extracts on biofilm formation by *C. albicans* and *C. krusei* on glass surfaces (OD reading; n=3). For each strain, the values labelled with dissimilar letters indicate significant differences in biofilm development after different types of tea treatment ($p<0.05$).

493 **Fig. 5** Correlation between cell surface hydrophobicity (arcsine-transformed) and attachment
494 (OD reading) upon tea treatments plotted using binomial regression. (A) *C. krusei* 27B and
495 (B) *C. krusei* 37B.

496

497 **Fig. 6** SEM micrographs of control (untreated) *C. krusei* 37B biofilm (A: at 2,000 ×
498 magnification; B: at 5,000 × magnification; scale bar: 20 μm), green tea extract treated *C.*
499 *krusei* 37B biofilm (C: 2,000 × magnification; D: 5,000 × magnification; scale bar: 20 μm);
500 and confocal laser scanning microscopic images of untreated *C. krusei* 37B biofilm (E; scale
501 bar: 50 μm), green tea extract treated *C. krusei* 37B biofilm (F; scale bar: 10 μm), and pu-erh
502 tea extract treated *C. krusei* 37B biofilm (G; scale bar: 10 μm). Red arrows indicate the cells
503 with indentations (live-dead stain; yellow blastospores/cells indicate dead cells as opposed to
504 the live cells which are green).

505